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# Function of the 23 kDa extrinsic protein of Photosystem II as a manganese binding protein and its role in photoactivation

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**Abstract**

The function of the extrinsic 23 kDa protein of Photosystem II (PSII) was studied with respect to Mn binding and its ability to supply Mn to PSII during photoactivation, i.e. the light-dependent assembly of the tetramanganese cluster. The extrinsic proteins and the Mn cluster were removed by TRIS treatment from PSII-enriched membrane fragments and purified by anion exchange chromatography. Room temperature EPR spectra of the purified 23 kDa protein demonstrated the presence of Mn. Photoactivation was successful with low Mn concentrations when the 23 kDa protein was present, while in its absence a higher Mn concentration was needed to reach the same level of oxygen evolution activity. In addition, the rate of photoactivation was significantly accelerated in the presence of the 23 kDa protein. It is proposed that the 23 kDa protein plays an important role in providing Mn during the process of PSII assembly and that it acquires Mn during the light-induced turnover of D1 in the PSII damage–repair cycle and delivers Mn to repaired PSII.


*Keywords:* Photosynthesis; Photosystem II; Photoactivation; 23 kDa protein; Manganese

## 1. Introduction

Photosystem II (PSII) is the protein complex in thylakoid membranes catalyzing the oxidation of water and the reduction of plastoquinone. The oxidation of water involves a tetramanganese cluster bound to the reaction center protein D1 [1,2]. In addition to the Mn cluster, the cofactors $Ca^{2+}$ and $Cl^{-}$ are obligatorily required for photosynthetic water splitting activity, their exact function is still unknown [3,4]. Three extrinsic proteins with the molecular masses of 33, 23 and 17 kDa (PsbO, PsbP, PsbQ) are bound to the lumenal side of PSII. The 33 kDa protein is most strongly bound and stabilizes the Mn cluster. The 17 kDa protein has been observed to increase the binding affinity of $Cl^{-}$ to PSII and the 23 kDa protein to be involved in increasing the binding affinities of both $Cl^{-}$ and $Ca^{2+}$, in addition to their general function in stabilizing the Mn cluster (for review see [5]). The structures of the extrinsic proteins are known while their function remains obscure [2,6,7].

The final step in the assembly of PSII during chloroplast development is the light-dependent ligation of the Mn cluster, a process called photoactivation [8–11]. In some organisms (green algae and gymnosperms), the PSII holoenzyme is synthesized independently of light, while the assembly of the functional Mn cluster occurs subsequently and requires illumination [12]. Even in mature leaves it is expected that PSII centers exist for a certain time in an inactive state in which the Mn cluster is absent. The D1 protein of PSII has a very high turnover rate, so that under all circumstances, a certain percentage of PSII centers

*Abbreviations:* Chl, chlorophyll; DCPIP, 2,6-dichlorophenol-indophenol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; MES, 4-morpholineethanesulfonic acid; PSII, Photosystem II; SDS-PAGE, sodium dodecyl sulfate polyacrylamid gel electrophoresis; TRIS, Tris(hydroxymethyl)aminomethane; TyrZ, redox active amino acid residue of the D1 protein

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is in the state of disassembly and reassembly. Before the D1 protein can be degraded, the extrinsic proteins and the Mn cluster have to be released [13,14].

The loss of the D1 protein is stimulated by photoinhibitory illumination (for review see [15,16]).

A question arises concerning the fate of Mn during the damage–repair cycle. Is Mn released in the form of free $Mn^{2+}$-ions into the lumen or is it sequestered by binding to proteins? In the first case the rebinding of $Mn^{2+}$ to PSII would be diffusion-limited while more sophisticated mechanisms must be envisaged if there were the involvement of a "Mn storage protein" capable in binding and delivering $Mn^{2+}$ in a controlled way to PSII during photoactivation.

Nievelstein et al. [17] reported on a diaphorase from *Narcissus pseudonarcissus* chromoplasts. This diaphorase was organized as a homodimer of the extrinsic 23 kDa protein of PSII. In its EPR spectrum, this 23 kDa dimer showed a $g$=4 signal which may be indicative for manganese. We hypothesized that the 23 kDa protein may play a role in Mn binding during the turnover of the D1 protein.

To model this step during the PSII damage–repair cycle we used TRIS-washed PSII particles which are known to be depleted of the Mn cluster and of the extrinsic proteins. After this treatment the water soluble fraction contained the extrinsic proteins. After purification by anion exchange chromatography their Mn content was determined by room temperature EPR spectroscopy. Photoactivation assays showed that fractions containing the 23 kDa protein were capable of performing very effectively the photoactivation reaction. The results indicate an important role of the 23 kDa protein as a manganese storage protein and in photoactivation.

## 2. Materials and methods

### 2.1. Preparation of PSII particles

PSII-enriched membrane fragments from spinach were prepared according to [18] with modification as described in [19]. The activity of the samples was approximately 500 μmol $O_2$ (mg of Chl)$^{-1}$h$^{-1}$.

### 2.2. NaCl treatment

PSII particles from spinach were treated with 1.5 M NaCl in the dark according to [20] to release the 23 kDa and 17 kDa proteins.

### 2.3. $CaCl_2$ treatment

PSII particles from spinach were treated with a buffer containing 1 M $CaCl_2$, 0.4 M sucrose, 50 mM MES (pH 6.0) for 30 min in the dark (Chl concentration: 0.5 mg/ml) to release all three extrinsic proteins. After this treatment the samples were washed twice with a buffer containing 0.4 M sucrose, 15 mM NaCl, and 50 mM MES (pH 6.5).

### 2.4. $NH_2OH$ treatment

Mn depletion was carried out by incubating NaCl-treated PSII particles from spinach at a concentration of 0.5 mg Chl/ml in a buffer containing 5 mM $NH_2OH$, 400 mM sucrose, 15 mM NaCl, and 50 mM MES (pH 6.5) for 1 h in the dark on ice, followed by two washes with the same buffer without $NH_2OH$. After $NH_2OH$ treatment without previous NaCl treatment about 90% of the 33 and 23 kDa protein remained bound to PSII while about 50% of the 17 kDa protein was released into the supernatant, as estimated by SDS PAGE.

### 2.5. TRIS treatment

Mn and extrinsic proteins were depleted by incubating PSII particles at a concentration of 0.5 mg Chl/ml in a buffer containing 0.5 M TRIS (pH 8.4) for 10 min in the light on ice, followed by a centrifugation at 43000×$g$. The supernatant was stored at −20 °C for further purification; the pellet was washed twice with a buffer containing 300 mM sucrose, 10 mM NaCl, 50 mM MES (pH 6.5). After TRIS treatment at least 30% of the 33 kDa protein remained bound to PSII while almost all of the 23 and 17 kDa protein was released to the supernatant, as estimated by SDS PAGE.

### 2.6. Anion exchange chromatography

TRIS supernatant was dialyzed against 50 mM TRIS/HCl (pH 8.0) as a starting buffer and applied to a Mono Q column (Pharmacia Biotech). The 17 kDa protein was not retarded, the 23 and 33 kDa proteins were eluted with a linear NaCl gradient of 0–0.35 M in starting buffer at a flow rate of 1 ml/min. The samples containing the pure extrinsic proteins were dialysed against a buffer containing 300 mM sucrose, 10 mM NaCl, 50 mM MES (pH 6.5). Protein quantification was done photometrically using an extinction coefficient of 19721 (M cm)$^{-1}$ for the 33 kDa, 24924 (M cm)$^{-1}$ for the 23 kDa and 14711 (M cm)$^{-1}$ for the 17 kDa protein, determined from the amino acid sequence by using an algorithm available at http://paris.chem.yale.edu/extinct.html.

### 2.7. SDS-PAGE and Western blotting

SDS-gel electrophoresis was carried out in 15% polyacrylamide gels according to [21]. Semidry Western blotting was performed using a Multiphor II Novablot Unit (Pharmacia Biotech). The proteins were identified by using polyclonal antibodies raised against each, the 23 kDa, 33 kDa and 17 kDa proteins. In addition, antibodies against FNR were used. For detection the ECL-system (Amersham) was used according to the manufacturer's protocol.

### 2.8. Recombinant 23 kDa protein

Expression and purification of the 23 kDa protein were as described in [22]; the *E. coli* strain was kindly provided by A. Seidler (Ruhr-Universität Bochum, Germany).

### 2.9. EPR measurements

X-band room temperature EPR spectra were recorded with a Bruker 300 spectrometer at 9.8 GHz microwave frequency, 63 mW microwave power and 100 kHz modulation frequency. The samples were acidified for visualisation of the $Mn^{2+}$-signal with $H_2SO_4$ (final concentration 1 M). In the presence of 0.5 M TRIS (pH 8.4), EPR signals of $MnCl_2$ standard solutions were smaller than in water or phosphate buffer.

### 2.10. Photoactivation

Photoactivation was conducted in a buffer containing 400 mM sucrose, 15 mM NaCl, and 50 mM MES (pH 6.5) using a final Chl concentration of 250 μg $ml^{-1}$. Samples were incubated at room temperature and illuminated with white light, using an intensity of 30 μmol quanta $m^{-2}s^{-1}$ in the presence of 0–200 μM $MnCl_2$, 50 mM $CaCl_2$ and 6 μM DCPIP as electron acceptor. When photoactivation assays were performed in the presence of the extrinsic proteins, the proteins were preincubated with the actual $MnCl_2$ concentration at room temperature for 5 min before PSII and the other additives were added. The ratio between PSII and the extrinsic proteins was 1:1.

### 2.11. $O_2$-evolution measurements

PSII activity was measured with a Clark-type oxygen electrode at pH 6.5 using 1 mM *p*-phenylbenzoquinone as electron acceptor.

## 3. Results

### 3.1. Isolation and characterization of a Mn-containing 23 kDa protein

The extrinsic proteins and the manganese cluster were released by subjecting PSII-enriched membrane fragments to TRIS treatment. The proteins of the supernatant were separated by anion exchange chromatography (Fig. 1A), characterized by SDS-PAGE (Fig. 1B) and their Mn content was measured by X-band EPR spectroscopy at room temperature (Fig. 1C). As shown in Fig. 1, the supernatant of the TRIS-treated sample contained several prominent bands (lane 4), among which the 17, 23 and 33 kDa proteins were identified according to their molecular masses and by the use of specific antibodies. The supernatant of the TRIS-treated sample was purified by anion exchange chromatography yielding three fractions which contained the proteins of interest: The non-binding fraction contained the 17 kDa protein (Fig. 1B, lane 5). A fraction containing the 23 kDa protein eluted with 77 mM NaCl (Fig. 1, lane 2). At 100 mM NaCl, the 33 kDa protein and a protein with higher molecular mass, identified as FNR, were recovered (Fig. 1B, lane 1).

To address the question of whether manganese is bound to the extrinsic proteins, EPR measurements were performed at room temperature. As shown in Fig. 1C, no EPR signal was detectable in the supernatant of TRIS-treated PSII-particles (Fig. 1C, spectrum a). However, denaturation by the addition of $H_2SO_4$ led to the appearance of six lines, the characteristic signal for $Mn^{2+}$ (Fig. 1 C, b). The size of the signal corresponded to 6 μM $Mn^{2+}$ at a protein concentration corresponding to 0.5 mg Chl/ml. Some residual Mn remained bound to PSII, since the pelleted particles after TRIS treatment showed a small EPR signal after the addition of $H_2SO_4$ (data not shown). When the Mn content was tested with the individual purified proteins, only the fraction containing the 23 kDa protein showed a manganese signal after the addition of $H_2SO_4$ (Fig. 1C, c). Both fractions which contained the isolated 33 kDa or 17 kDa protein, respectively (Fig. 1 C, d, e), were negative in this respect. The Mn content of the purified 23 kDa protein was lower than in the original TRIS supernatant which needed to be dialyzed prior to anion exchange chromatography. This led to a significant loss of Mn (between 60% and 70%), indicating that most of the Mn was weakly bound to proteins or other components. It is known from other proteins that the binding of $Mn^{2+}$ is often very unstable and can result in a complete loss of $Mn^{2+}$ during the isolation procedure [23].

As was the case for acidification, the addition of $NH_2OH$ or dithionite to the Mn-containing 23 kDa protein led to a release of $Mn^{2+}$ and a signal of comparable size was obtained (data not shown). An ambient midpoint potential for the Mn release of approximately 100 mV was determined by redox titration of the EPR signal. This suggests that ligands which are involved in the coordination of $Mn^{2+}$ were reduced. Alternatively, the rather unlikely possibility that Mn is coordinated in a higher oxidation state to the 23 kDa protein and that it is released after reduction to $Mn^{2+}$ must be considered. Unfortunately, Mn bound to the protein could not be detected by X-band EPR spectroscopy even at liquid helium temperatures. It is known that the appearance of a $Mn^{2+}$ signal depends on the geometry of the ligands (e.g. [24]).

To confirm the capacity of the 23 kDa protein to bind Mn, we used the recombinant protein. It was possible to incorporate $Mn^{2+}$ into the overexpressed 23 kDa protein. In fact, its capacity in this respect was very considerable as confirmed by EPR spectroscopy. As shown in Fig. 2, no EPR signal was seen when 20 μM $MnCl_2$ was added to the recombinant protein (2 μM), indicative of coordination. EPR signals appeared only at concentrations exceeding 30

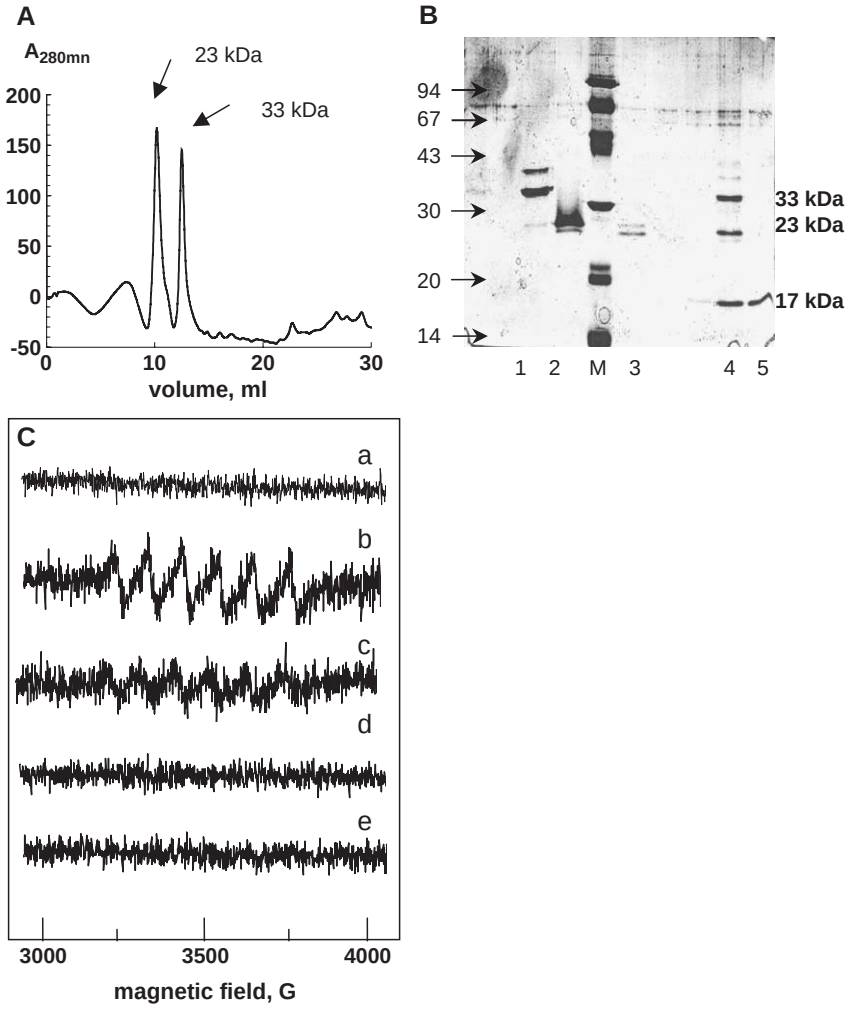


Fig. 1. Anion exchange chromatogram (A) SDS-PAGE (B) and EPR spectra of the different preparations of the extrinsic proteins. (A) Anion exchange chromatography. Samples were eluted by applying an NaCl-gradient (0–350 mM). (B) SDS-PAGE showing the different fractions after anion exchange chromatography: 1, 33 kDa protein plus FNR; 2, 23 kDa protein; M, molecular weight marker; 4, supernatant after TRIS-washing prior to chromatography; 5, 17 kDa protein, non-binding fraction. (C) (a) Supernatant of TRIS-treated PSII particles corresponding to 0.5 mg Chl/ml with no addition; (b) supernatant of TRIS-treated PSII particles with 1 M $H_2SO_4$; (c) isolated 23 kDa protein with 1M $H_2SO_4$; (d) isolated 33 kDa protein with 1 M $H_2SO_4$; (e) isolated 17 kDa protein with 1 M $H_2SO_4$. EPR spectra were recorded at room temperature using a flat cell.

μM (data not shown). Only small amounts of Mn were found to bind to the purified 33 kDa protein while the 17 kDa protein did not show any binding of Mn (Fig. 2).

### *3.2. Stimulation of photoactivation of Mn-depleted PSII by the 23 kDa protein*

To investigate the involvement of the extrinsic proteins in the photoactivation of Mn-depleted PSII, oxygen evolution was measured. Fig. 3 shows photoactivation experiments performed with NaCl/$NH_2OH$-washed PSII particles. This procedure led to the removal of the manganese cluster and a selective loss of the 23 kDa and 17 kDa proteins, leaving the 33 kDa protein unaffected (data not shown). It was necessary to have the 33 kDa protein attached to PSII because it serves as an attachment site for the other extrinsic proteins [25]. With this material, photoactivation studies were carried out comparing the effects of free $Mn^{2+}$ with the effects of the 23 kDa protein containing bound Mn. By adding saturating concentrations (200 μM $MnCl_2$) a comparable yield of photoactivation was obtained both in the presence and the absence of the 23 kDa protein. However, at low $MnCl_2$ concentrations a clear stimulation was seen in the presence of the 23 kDa protein (see inset Fig. 3). This indicates that the 23 kDa protein facilitates assembly of the Mn cluster, most likely by providing Mn to the donor side of PSII. The Mn-containing 23 kDa protein as purified by anion exchange chromatography could not be used directly because of the high pH value (pH 8.0) of this sample. This necessitated dialysis and concentration, accompanied by a loss of bound Mn (as revealed by EPR). Therefore we reconstituted the purified or the recombinant 23 kDa protein with the Mn concentrations given in Fig. 3 before adding PSII particles to start photoactivation. As shown in Fig. 2, the addition of low concentrations of $Mn^{2+}$ did not result in an EPR spectrum. When photoactivation assays were

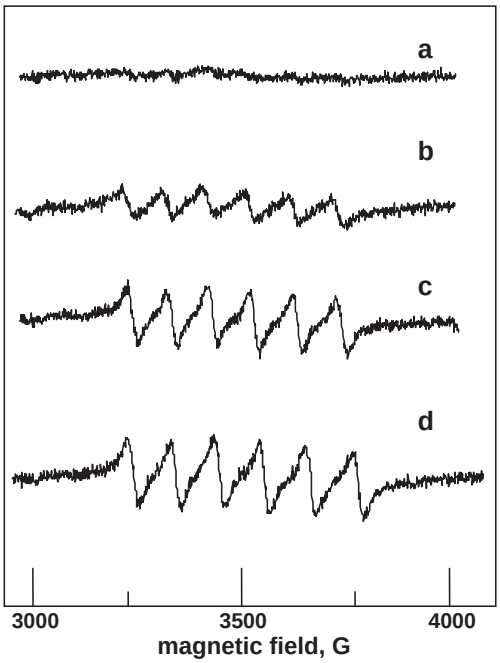


Fig. 2. Binding of $Mn^{2+}$ to the extrinsic proteins. The EPR spectra for the (a) 23 kDa protein (b) 33 kDa protein (c) 17 kDa protein were recorded using 2 μM protein and 20 μM $MnCl_2$ in 5 mM MES (pH 6.5). Panel (d) represents the spectrum of the buffer containing 20 μM $MnCl_2$. EPR spectra were recorded at room temperature using a flat cell.

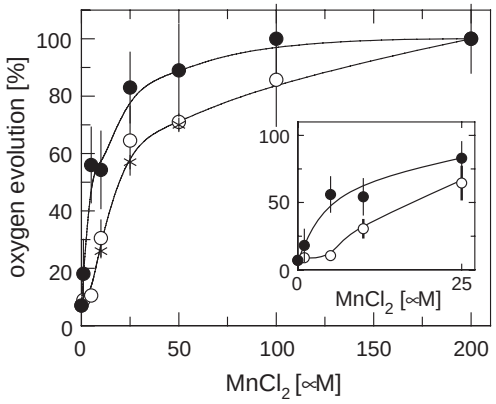


Fig. 3. Photoactivation of Mn-depleted PSII particles. Dependence on the Mn concentration in the presence (●) and absence (○) of the 23 kDa protein. The indicated Mn concentrations were added to the 23 kDa protein and incubated for 5 min before adding it to the photoactivation assay. Asterisk, photoactivation in the presence of the 17 kDa protein. The extrinsic proteins were added at a final concentration of 1 μM, the concentration of PSII was 1 μM, assuming 250 Chl per reaction center. The photoactivation of the NaCl/$NH_2OH$-washed PSII particles (250 μmol Chl/ml) was performed under illumination for 20 min with white light (30 μmol quanta $m^{-2}s^{-1}$) in the presence of 50 mM $CaCl_2$, 6 μM DCPIP and the indicated $MnCl_2$ concentration. Oxygen evolution was measured in the presence of 1 mM *p*-phenylbenzoquinone. The maximum activity was 500 μmol $O_2$ mg $Chl^{-1}$ $h^{-1}$ prior to the NaCl/$NH_2OH$ treatment; the maximum activity after photoactivation was between 110 and 230 μmol $O_2$ $Chl^{-1}$ $h^{-1}$, depending on the material used. The average value of four independent measurements is given; the error bars show the standard deviation.

performed in the presence of the 23 kDa protein without preloading with Mn, a much smaller effect on the yield of photoactivation was observed (data not shown).

When the 17 kDa protein was added instead of the 23 kDa protein under otherwise identical conditions, no stimulation of photoactivation was observed. When both proteins, 23 and 17 kDa, were added, no additional stimulatory effect was found (data not shown).

The evidence presented supports the idea that the 23 kDa protein functions in the delivery of manganese to the donor side of Photosystem II. Alternatively, it appears conceivable that the function of the 23 kDa protein is rather to protect oxidized Mn bound to the donor side against reductants. To test for this possibility, we followed the time course of photoactivation under three different conditions: first, with samples illuminated in the presence of the Mn-containing 23 kDa protein (● in Fig 4); second, with samples which were photoactivated in the absence of the 23 kDa protein (○); and third, with samples to which the 23 kDa protein in its manganese-free form was added immediately after switching off the photoactivation light (△). As can be seen in Fig. 4, photoactivation was achieved very fast and with a high efficiency in the presence of the Mn-containing 23 kDa protein. In contrast, the addition of the manganese-free 23 kDa protein after the photoactivation period had no significant effect on the rate and the yield of photoactivation. This indicates that the 23 kDa protein functions to provide

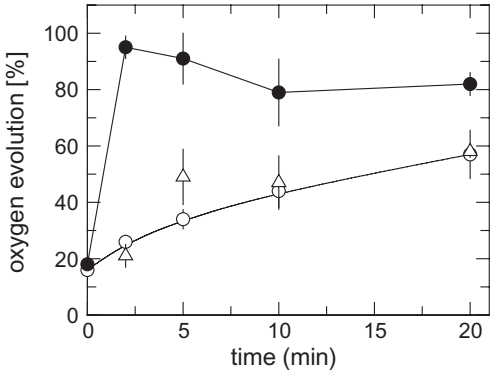


Fig. 4. Time course of the photoactivation of Mn-depleted PSII particles. The time course of photoactivation in the presence (closed circles) and absence (open symbols) of the Mn-containing 23 kDa protein; triangles, photoactivation was performed in the absence of the 23 kDa protein and the Mn-free 23 kDa protein was added in the dark immediately after turning off the photoactivation light. The photoactivation of the NaCl/$NH_2OH$-washed PSII particles (1 μM, assuming 250 Chl per reaction center) was performed under illumination with white light (30 μmol quanta $m^{-2}s^{-1}$) for a given time in the presence of 50 mM $CaCl_2$, 6 μM DCPIP and 5 μM $MnCl_2$. In the case of photoactivation in the presence of the Mn-containing 23 kDa protein, the 23 kDa protein was incubated with 5 μM $MnCl_2$ for 5 min before adding it to the photoactivation assay. The final concentration of the 23 kDa protein was 1 μM. Oxygen evolution was measured in the presence of 1 mM *p*-phenylbenzoquinone. The maximum activity after photoactivation was 115 μmol $O_2$ mg $Chl^{-1}$ $h^{-1}$, when photoactivation was performed in the presence of 200 μM $MnCl_2$. In the presence of 5 μM $MnCl_2$, the maximum activity was 68 μmol $O_2$ mg $Chl^{-1}$ $h^{-1}$. This value was set to 100%. The average value of four independent measurements is given; the error bars show the standard deviation.

Mn thereby facilitating the photoactivation process rather than ensuring the stability of the assembled Mn cluster.

When photoactivated samples were incubated in the dark over a longer time period (5–10 min), the loss of oxygen evolution was faster in the absence than in the presence of the 23 kDa protein (data not shown). Thus there seems to be an additional effect which is in fact related to the stabilization of the assembled Mn cluster.

In addition, the effect of the 33 kDa protein on the Mn requirement for photoactivation was investigated. $CaCl_2$/$NH_2OH$-treated PSII samples were used for this purpose which were deprived of all extrinsic proteins and the Mn cluster. As in the case of reconstitution with the Mn-containing 23 kDa protein, Mn was added to the 33 kDa protein before adding PSII and starting the photoactivation assay. No difference in the yield of photoactivation was observed in the absence of the 33 kDa protein or in its presence (data not shown).

## 4. Discussion

TRIS-treatment of PSII particles not only leads to a release of Mn and of the extrinsic proteins, but also to the concomitant binding and sequestration of Mn by the 23 kDa protein (Fig. 1). The stoichiometry of Mn uptake by the 23 kDa protein was determined to be 1:10 when measured by EPR spectroscopy (Fig. 2). However this value should be regarded with caution because some of the binding may be unspecific. The 23 kDa protein contains a large number of acidic amino acid residues [6] which may ligate Mn unspecifically.

Photoactivation assays showed that the manganese-containing 23 kDa protein can restore oxygen evolution of Mn-depleted PSII particles very effectively in the absence of free Mn (Figs. 3,4). There are earlier reports on the occurrence of Mn containing proteins involved in photosynthetic oxygen evolution [26,27]. A 65 kDa protein which contained Mn and which was able to restore oxygen evolution activity in Mn-depleted membranes was thought to be important. However, Abramowicz and Dismukes [28] showed that this protein was the β-subunit of the ATP synthase cF1 sub-complex. There are also reports describing a Mn-containing 33 kDa protein isolated from oxygen-evolving PSII preparations [25,27]. Abramowicz and Dismukes [27] isolated the extrinsic 33 kDa protein from PSII, which contained two Mn per protein (see also [29]). However the reconstitution of oxygen evolving activity using this purified Mn-containing 33 kDa protein failed. This observation is in accordance with our results since the photoactivation of $CaCl_2$/$NH_2OH$-treated samples failed using the purified 33 kDa protein and low concentrations of $MnCl_2$. Instead, when these authors [28] used a crude protein mixture containing all three extrinsic proteins, 25% of the original oxygen evolution activity was restored. Our results allow the interpretation that the 23 kDa protein present in their crude extract was responsible for this observation.

We suggest that the Mn-containing 23 kDa protein has an important function during the assembly and the turnover of PSII. It acts as a Mn storage protein, capable of delivering Mn to the D1 protein in the photoactivation step. This role is particularly important during the development of the photosynthetic electron transport chain in greening tissues. During de novo synthesis of PSII, the assembly of the Mn cluster is the last step to occur [30]. A second function of the 23 kDa protein could be its involvement in the damage–repair cycle of PSII (see [15,16]). Here, the D1 protein is first tagged through a photoinhibitory event and in a second step, the extrinsic proteins [13] and the Mn [14] are released before the D1 protein is degraded. The 23 kDa protein may accommodate the liberated Mn. After synthesis of a new D1 protein and assembly of the PSII reaction centre, the process of photoactivation may involve the Mn-containing 23 kDa protein.

The following scenario for the photoactivation process can be imagined (under the assumption that the 23 kDa protein contains at least two Mn):

1. The Mn-containing 23 kDa protein comes into contact with the donor side of PSII.
2. In the first light-dependent step, $Tyr_Z$ is oxidized, Mn is released from the 23 kDa protein and one $Mn^{2+}$ is oxidized by $Tyr_Z^+$ and bound to PSII. $Mn^{3+}$ has a higher affinity for PSII than $Mn^{2+}$. A second $Mn^{2+}$ interacts with $Mn^{3+}$ and the first instable intermediate of the Mn cluster is formed (for current models of photoactivation see [8,9]).
3. The 23 kDa protein is now tightly bound to the donor side of PSII leading to a protection of the site of assembly and to a stabilization of the first intermediate ($Mn^{3+}$–$Mn^{2+}$).
4. In the second light-dependent step, the second $Mn^{2+}$ is oxidized and the final tetramanganese cluster can be assembled.

It is a known that the 23 kDa protein plays a role in the stabilization of the Mn cluster, once finally assembled. It is also known that Mn-binding to PSII is necessary for a tight binding of the 23 kDa protein [31]. Here we provide evidence that is has an additional important function in delivering Mn to PSII and during the assembly of the Mn cluster.

The FUD 39 mutant of *Chlamydomonas reinhardtii* lacks the 23 kDa protein but possesses the 17 and 33 kDa proteins [32]. This mutant has a decreased capacity to perform photoactivation and is more susceptible to photoinhibition than the wild-type [33,34]. Photoactivation in the FUD 39 mutant is much slower than in the wild-type. Data from this mutant are similar to our observation (Fig. 4) in that 50% activity is observed after 7–8 min and maximum activity after 25 min. In the wild-type, 1–2 min for 50% and 10 min for 100% activation were needed [33].

It has been proposed that this mutant has a decreased $Cl^-$ affinity and needs a longer dark period between the first step (assembly of 2 Mn) and the second step (assembly of the complete tetramanganese cluster) of photoactivation. In the light of our data, we interpret this observation as an indication that the 23 kDa protein indeed stabilizes the first intermediate of photoactivation.

The situation in cyanobacteria is unclear since there is no indication of the extrinsic 12 kDa protein (PsbU) and cytochrome $c_{550}$ (PsbV) participating in manganese binding. Cyanobacteria might not need such a Mn storage protein. The Mn concentration inside the cells might be much higher than in the chloroplast and the manganese exchange with the surroundings is facilitated [35]. However, there is a recent report that homologs of the 23 kDa (PsbP) and the 17 kDa (PsbQ) proteins are expressed in addition to PsbV and PsbU [36]. PsbP was present in substoichiometric amounts (only 3% of the PSII centres had a copy of PsbP) while one copy of PsbQ was present per PSII. Mutants of *psbP* and *psbQ* exhibited reduced photoautotrophic growth and decreased water oxidation activity when grown under $CaCl_2$ depletion. It was suggested that these proteins play a regulatory role in providing PSII with calcium and chloride. Furthermore it was proposed that PsbP may be required for the assembly of PSII. This proposal fits nicely with our suggestion on the role of the 23 kDa protein during photoactivation.

In conclusion, we suggest that the 23 kDa protein is a Mn storage protein in plants and has an important function in delivering $Mn^{2+}$ to PSII during the assembly of de novo synthesized and/or repaired PSII. It seems to facilitate the assembly of the tetramanganese cluster in vivo. However, its absence is not lethal but slows down the process of photoactivation. Investigations of the nature of the Mn binding site in the 23 kDa protein, on the binding affinity, the stoichiometry and on its involvement in the uptake of Mn liberated upon photoinhibition are required to substantiate this model.

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